Mechanisms of Carcinogenesis: Contributions of Molecular Epidemiology
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Genetic Pathways of Two Types of Gastric Cancer

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Summary

Multiple genetic and epigenetic alterations in oncogenes, tumour-suppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes and genetic instability as well as telomerase activation are implicated in the multistep process of human stomach carcinogenesis. However, particular combinations of these alterations differ in the two histological types of gastric cancer, indicating that well-differentiated or intestinal-type and poorly differentiated or diffuse-type carcinomas have distinct carcinogenetic pathways. In the multistep process of well-differentiated-type carcinogenesis, the genetic pathway can be divided into three subpathways: an intestinal metaplasia→adenoma→carcinoma sequence, an intestinal metaplasia→carcinoma sequence and *de novo*. In the multistep process of well-differentiated-type or intestinal-type gastric carcinogenesis, infection with *Helicobacter pylori* may be a strong trigger for hyperplasia of hTERT-positive 'stem cells' in intestinal metaplasia. Genetic instability and hyperplasia of hTERT-positive stem cells precede replication error at the D1S191 locus, DNA hypermethylation at the D17S5 locus, *pS2* loss, *RARβ* loss, *CD44* abnormal transcripts and *p53* mutation, all of which accumulate in at least 30% of incomplete intestinal metaplasias. All of these epigenetic and genetic alterations are common events in intestinal-type gastric cancer. An adenoma→carcinoma sequence is found in about 20% of gastric adenomas with *APC* mutations. In addition to these events, *p53* mutation and loss of heterozygosity (LOH), reduced *p27* expression, *cyclin E* expression and the presence of c-*met* 6.0-kb transcripts allow malignant transformation from the above precancerous lesions to intestinal-type gastric cancer. *DCC* loss, *APC* mutations, 1q LOH, *p27* loss, reduced tumour growth factor (TGF)-β type I receptor expression, reduced *nm23* expression and c-*erb*B gene amplification are frequently associated with an advanced stage of intestinal-type gastric cancer. The de-novo pathway for carcinogenesis of well-differentiated gastric cancer involves LOH and abnormal expression of the *p73* gene that is responsible for the development of foveolar-type gastric cancers with pS2 expression.

On the other hand, LOH at chromosome 17p, mutation or LOH of *p53* and mutation or loss of E-cadherin are preferentially involved in the development of poorly differentiated gastric cancers. In addition to these changes, gene amplification of K-*sam*, and c-*met* and *p27* loss as well as reduced nm23 obviously confer progression, metastasis and diffusely productive fibrosis. Mixed gastric carcinomas composed of well-differentiated and poorly differentiated components exhibit some but not all of the molecular events described so far for each of the two types of gastric cancer.

Besides these genetic and epigenetic events, well-differentiated and poorly differentiated gastric cancers also organize different patterns of interplay between cancer cells and stromal cells through the growth factor/cytokine receptor system, which plays an important role in cell growth, apoptosis, morphogenesis, angiogenesis, progression and metastasis.

Meta-analysis of epidemiological studies and animal models show that both intestinal and diffuse types of gastric cancer are equally associated with *H. pylori* infection. However, *H. pylori* infection may play a role only in the initial steps of gastric carcinogenesis. Differences in *H. pylori* strain, patient age, exogenous or endogenous carcinogens and genetic factors such

as DNA polymorphism and genetic instability may be implicated in two distinct major genetic pathways for gastric carcinogenesis.

Introduction

Striking advances in molecular dissection of precancerous and cancerous lesions of the stomach indicate that genetic and epigenetic alterations in oncogenes, tumour-suppressor genes, DNA-repair genes, cell-cycle regulators, telomeres and telomerase, as well as genetic instability at microsatellite foci are involved in the multistep process of human stomach carcinogenesis (Sano *et al.*, 1991; Tahara, 1993; Tahara *et al.*, 1996a).

There are several histological classifications of gastric cancer. Lauren (1965) divided gastric cancer into two types, intestinal and diffuse, and the Japan Research Society for Gastric Cancer (JRSGC, 1999) classified it into five common types. The JRSGC classification is similar to that of the World Health Organization (Hamilton & Aaltonen, 2000). In this chapter, we use a two-type classification: the intestinal or well-differentiated type (which includes the papillary and tubular adenocarcinomas of the JRSGC classification), and the diffuse or poorly differentiated type (which includes the diffuse and signet-ring cell carcinomas of the JRSGC classification).

The genetic and epigenetic changes found in gastric carcinoma differ, depending upon the histological type of gastric cancer, indicating that different carcinogenetic pathways exist for intestinal and diffuse types of carcinomas (Table 1; Figures 1 and 2). In addition, cancer–stromal interaction through the growth factor/cytokine receptor system, which plays a pivotal role in morphogenesis, cancer progression and metastasis, is also much different between the two types of gastric carcinoma (Tahara *et al.*, 1993, 1994).

This chapter provides a detailed overview of the molecular machinery that underlies stomach carcinogenesis.

Oncogenes

Several proto-oncogenes, including c-*met*, K-*sam* and c-*erb*B2, are frequently activated in gastric carcinomas. The amplification of the c-*met* gene encoding a receptor for hepatocyte growth factor/scatter factor is found in 19% of intestinal and 39% of diffuse gastric cancers, frequently accompanied by diffusely productive fibrosis of the scirrhous type (Kuniyasu *et al.*, 1992). Most gastric carcinomas express two different c-*met* transcripts, one of 7.0 kb and the other of 6.0 kb. Expression of the 6.0-kb c-*met* transcript, which is expressed preferentially in cancer cells, correlates well with tumour staging, lymph node metastasis and depth of tumour invasion (Kuniyasu *et al.*, 1993). Soman *et al.* (1991) reported that the *tpr-met* rearrangement is expressed in gastric carcinomas and gastric precancerous lesions. However, we have not detected the *tpr-met* rearrangement in any gastric cancer or intestinal metaplasia.

The K-*sam* (KATO-III cell-derived stomach cancer amplified) gene has at least four transcriptional variants. Type II encodes a receptor for keratinocyte growth factor (Katoh *et al.*, 1992). Type II transcript is expressed only in carcinoma cells (not in cell lines from sarcomas). K-*sam* is preferentially amplified in 33% of advanced diffuse or scirrhous-type gastric carcinomas, but not in intestinal-type gastric carcinomas (Hattori *et al.*, 1990). Moreover, K-*sam* is never seen in esophageal or colorectal carcinomas. Gastric cancers that overexpress K-sam protein are associated with a less favourable prognosis.

In contrast to K-*sam*, c-*erb*B2 is preferentially amplified in 20% of intestinal gastric cancers but not in diffuse-type gastric cancer (Yokota *et al.*, 1988; Kameda *et al.*, 1990). Overexpression of c-*erb*B2 associated with gene amplification is closely correlated with a poor prognosis and liver metastasis (Oda *et al.*, 1990; Yonemura *et al.*, 1991). The amplification of c-*erb*B1 and c-*erb*B3 is found in 3% (Kameda *et al.*, 1990) and 0% (Katoh & Terada, 1993), respectively, of gastric cancers.

K-*ras* mutation is found in gastric intestinal metaplasias, adenomas and intestinal-type adenocarcinomas (Sano *et al.*, 1991; Lee *et al.*, 1995; Isogaki *et al.*, 1999), although its incidence is low (10–18%). However, K-*ras* mutation is not seen in diffuse-type gastric cancer. The *hst*-1 gene, isolated from a surgical specimen of human gastric cancer by the NIH/3T3 transformation assay, is rarely amplified in gastric cancer (2% of cases) (Yoshida *et al.*, 1988).

Table 1. Genetic and epigenetic alterations found in two types of gastric cancer

Genetic and epigenetic alterations	Incidence of cases with indicated alterations (%)	
	Well-differentiated[a]	Poorly differentiated[a]
Tumour suppressors		
p53 LOH, mutation	60	75
p73 LOH	53[b]	24
APC LOH, mutation	40–60	0
DCC LOH	50	0
LOH of chromosome 1q	44	0
LOH of chromosome 7q	53	33
LOH of chromosome 17q	0	40[c]
Loss of *pS2* expression	49	31
Loss of *RARβ*	64	0
Cell-cycle regulators		
Cyclin E amplification	33	7
Cyclin E overexpression	26	27
CDC25B overexpression	33	73
Loss of p16 expression	12	31
Loss of p27 expression	46	69
Oncogenes		
K-*ras* mutation	10	0
c-*met* amplification	19	39
K-*sam* amplification	0	33
c-*erbB2* amplification	20	0
Adhesion molecules		
E-cadherin mutation/loss	0	50
CD44 aberrant transcript	100	100
Microsatellite instability	20–40	20–70[c]
Histone deacetylation	61	82
Telomere/telomerase		
Telomere reduction	62	53
Telomerase activity	100	90
TERT expression	100	86

[a] According to the criteria of the JRSGC classification of gastric cancer
[b] Preferentially found in foveolar-type adenocarcinoma
[c] Preferentially found in patients younger than 35 years of age
LOH, loss of heterozygosity

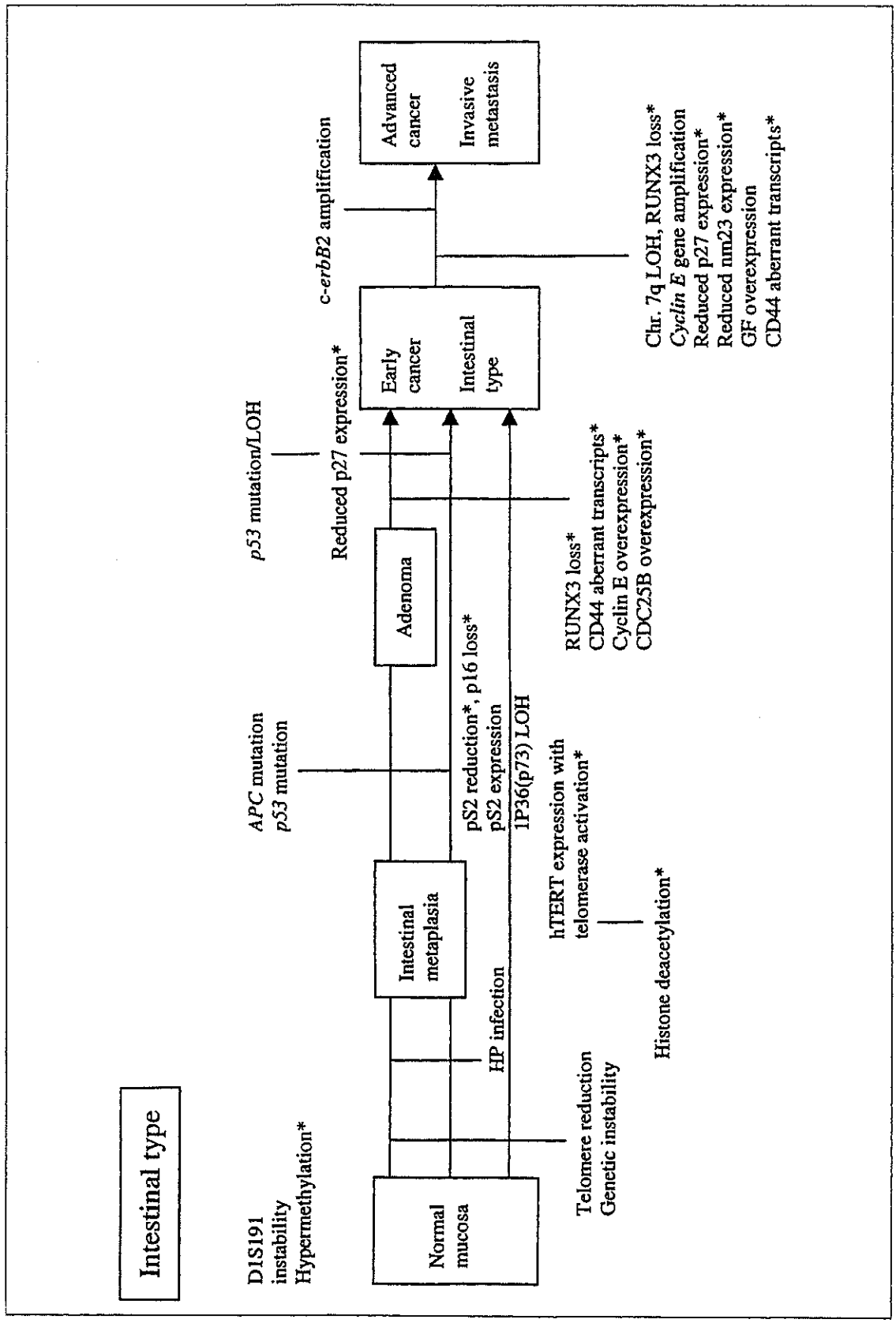

Figure 1. Multiple genetic and epigenetic alterations during human stomach carcinogenesis (intestinal type). * Epigenetic alterations. LOH, loss of heterozygosity; HP, *Helicobacter pylori*. From Tahara (2002)

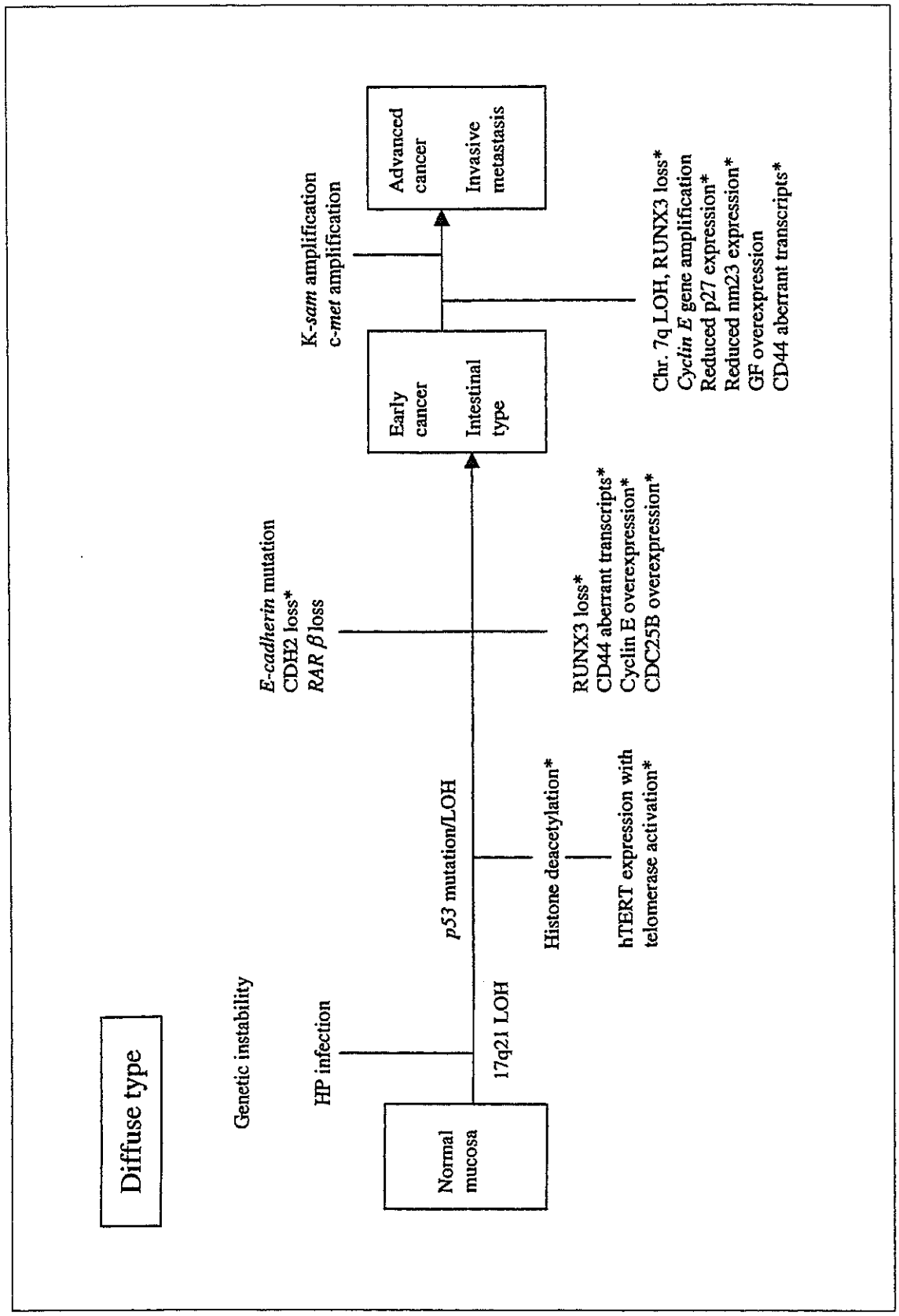

Figure 2. Multiple genetic and epigenetic alterations during human stomach carcinogenesis (diffuse type). * Epigenetic alterations. LOH, loss of heterozygosity; HP, *Helicobacter pylori*; GF, growth factor. From Tahara (2002)

Tumour suppressor genes

Alterations in the structure and function of tumour-suppressor genes, including *p53*, *p73*, *APC*, *DCC* and *FHIT*, are involved in stomach carcinogenesis. Among them, inactivation of the *p53* tumour-suppressor gene by LOH and mutation is the most frequent genetic event in gastric cancer, occurring in over 60% of gastric carcinomas regardless of histological type (Sano *et al.*, 1991; Tamura *et al.*, 1991; Yokozaki *et al.*, 1992). Alterations in the *p53* gene are also found in 13–37% of intestinal metaplasias and 33–58% of gastric adenomas or dysplasias (Tohdo *et al.*, 1993; Sakurai *et al.*, 1995; Ochiai *et al.*, 1996), indicating that *p53* gene mutation is an early event in stomach carcinogenesis. The mutation spectrum of this gene can serve as a marker of the effect of putative carcinogens (Harris, 1991). The mutation spectrum of the *p53* gene in gastric cancers in Hiroshima displays an intermediate pattern between those of colonic cancer and esophageal cancer (Uchino *et al.*, 1993; Maesawa *et al.*, 1995; Poremba *et al.*, 1995). *p53* Mutations at A:T sites are common in intestinal-type carcinomas; GC→AT transitions are predominant in diffuse-type carcinomas (Yokozaki *et al.*, 1992). Carcinogenic *N*-nitrosamines, which cause mainly GC→AT base substitutions, are found in many foods and can also be produced from amines and nitrates in the acidic environment of the stomach (Sugimura *et al.*, 1970; Mirvish, 1971).

LOH of the *p73* gene, a newly discovered tumour-suppressor gene related to *p53*, is detected in 38% of gastric cancers, especially intestinal adenocarcinomas that exhibit papillary structure similar to foveolar epithelium and express the pS2 trefoil factor (Yokozaki *et al.*, 1999a).

This type of gastric cancer with *p73* LOH shows allele-specific expression of *p73* but no gene mutation in the remaining allele. In addition, the incidence of *p53* abnormalities is low (25%). These observations indicate that LOH and abnormal expression of the *p73* gene may play a large role in the genesis of foveolar-type gastric adenocarcinoma, although this is not in line with Knudson's classic 'two-hit' model of carcinogenesis. We have already reported that 25% of intestinal gastric cancers show LOH on chromosome 1p by restriction fragment length polymorphism analysis using the MS1 (1p33–p35) probe (Sano *et al.*, 1991). However, these loci are rather centromeric when compared with the mapped region of the *p73* gene (1p36–33).

APC is a tumour-suppressor gene that is a susceptibility factor for familial polyposis coli (Kinzler *et al.*, 1991). Mutations in the *APC* gene also take place in gastric cancers and sporadic colorectal cancers. Interestingly, more than 50% of intestinal-type gastric cancers harbour *APC* mutations, whereas diffuse-type gastric cancers have none. Moreover, there is a distinct difference in the nature of *APC* mutations between gastric and colorectal cancers: missense mutation is dominant in gastric cancer whereas nonsense and frameshift mutations are common in colorectal cancers (Nakatsuru *et al.*, 1992). Somatic mutations of the *APC* gene are also seen in 20–40% of gastric adenomas and 6% of incomplete intestinal metaplasias (Nakatsuru *et al.*, 1993; Nishimura *et al.*, 1995). *APC* alteration is viewed as an early genetic event in the pathogenesis of intestinal-type gastric cancers (Yokozaki *et al.*, 1997). LOH at the *DCC* locus also is one of the characteristics of intestinal-type gastric cancer and is seen in 50–60% of primary gastric cancers (Sano *et al.*, 1991; Uchino *et al.*, 1992).

The hypothesis that *FHIT* gene alterations are involved in the development of primary gastric cancer remains controversial. Huebner's group reported the rearrangement of the *FHIT* gene, aberrant transcripts or both in 53% of primary gastric cancers and loss of FHIT protein in 67% (Ohta *et al.*, 1996; Baffa *et al.*, 1998). Chen *et al.* (1997a) demonstrated that aberrant transcripts were found not only in 46% of gastric cancers but also in 30% of non-cancerous gastric mucosas. Other studies showed that 13–16% of primary gastric cancers shared LOH of the *FHIT* gene and no abnormal transcripts (Tamura *et al.*, 1997; Noguchi *et al.*, 1999), although four of seven gastric cancer cell lines exhibited LOH of the *FHIT* gene (Tamura *et al.*, 1997). *FHIT* gene alterations and loss of FHIT protein should be evaluated in series involving many cases of gastric cancer and precancerous lesions to determine whether environmental factors or putative carci-

nogens are associated with differences between countries in frequency of FHIT abnormalities.

Several distinct chromosomal loci are deleted in gastric cancers. LOH at 1q and 7q are frequently associated with intestinal gastric cancer, whereas loss of 1p is relatively common in advanced diffuse gastric cancer (Sano *et al.*, 1991). Moreover, LOH at the *bcl-2* gene locus is seen in many intestinal gastric cancers and colorectal cancers (Ayhan *et al.*, 1994). Our deletion mapping study on 7q shows that LOH at the D7S95 locus correlates well with peritoneal dissemination (Kuniyasu *et al.*, 1994a). Recently, investigators in a study on allelic loss in xenografted human gastric carcinomas reported a high degree of allelic loss on several chromosomal arms in 18 xenografted gastric adenocarcinomas: 3p (81%), 4p (64%), 5q (69%), 8p (57%), 13q (59%), 17p (80%) and 18q (61%) (Yustein *et al.*, 1999). From these assigned loci, candidates for the tumour-suppressor gene responsible for stomach carcinogenesis may be identified in the future.

pS2, a gastric-specific trefoil factor normally expressed in the gastric foveolar epithelial cells, may function as a gastric-specific tumour suppressor, since the inactivation of the *pS2* gene by gene targeting causes dysplasia, adenoma and adenocarcinoma of the glandular stomach in mice (Masiakowski *et al.*, 1982; Lefebvre *et al.*, 1996). Recently, we found that the reduction or loss of the *pS2* gene by DNA methylation at the promoter region occurs in intestinal metaplasias and gastric adenomas. Conversely, 32% of gastric cancers display strong expression of the *pS2* gene and 40% of gastric cancers, especially the intestinal type, show no expression (Fujimoto *et al.*, 2000). Reduced expression or loss of the *pS2* gene by promoter methylation may play a role in the early stages of carcinogenesis of intestinal stomach carcinoma.

Recent in-vivo and in-vitro studies suggest that the nuclear retinoic acid receptor β (RARβ) functions as a tumour suppressor and that loss of *RARβ* by CpG promoter hypermethylation is associated with tumorigenesis (Lotan *et al.*, 1995; Seewaldt *et al.*, 1995; Hayashi *et al.*, 2001a). More recently, we found that hypermethylation of the *RARβ* gene promoter is preferentially observed in 64% of intestinal gastric cancers associated with reduced expression (Hayashi *et al.*, 2001b), but not in the diffuse type. Promoter hypermethylation is also detected in gastric intestinal metaplasia. Three gastric cancer cell lines (MKN-28, -45 and -74), all of which are derived from intestinal-type adenocarcinomas, exhibit a loss of *RARβ* expression by promoter methylation. *RARβ* expression is restored in these cell lines by 5-azacytidine or the histone deacetylase inhibitor trichostatin A. Overexpression of the RARβ in MKN-28 cells induces G_0–G_1 arrest, followed by down-regulation of the DNA methyltransferase 3α and DNA demethylase, and up-regulation of the acetylated histone H4. These results suggest that inactivation of RARβ as well as pS2 is implicated in gastric carcinogenesis of the intestinal type.

Cell-cycle regulators

Genetic and epigenetic abnormalities in cell-cycle regulators are involved in the development and progression of gastric cancer by causing unbridled proliferation. Most gastric cancers are associated with overexpression of positive regulators and reduction or loss of negative regulators, both of which co-operate to drive normal cells into malignancy.

The *cyclin E* gene is amplified in 15–20% of gastric carcinomas that are associated with its overexpression. The gene amplification or overexpression of cyclin E, or both cause aggressiveness and lymph node metastasis (Akama *et al.*, 1995). *Cyclin D1* gene amplification, on the other hand, is exceptional in gastric carcinomas but frequently occurs in esophageal carcinoma (Yoshida *et al.*, 1996).

CDC25 phosphatases dephosphorylate threonine and tyrosine residues at positions 14 and 15 in the cyclin-dependent kinases (CDKs) and then activate them (Honda *et al.*, 1993). Three types of CDC25 have been identified: CDC25A, -B and -C (Nagata *et al.*, 1991). CDC25A is expressed early in the G_1 phase of the cell cycle; CDC25B is expressed in both the G_1/S and G_2 phases (Jinno *et al.*, 1994) and CDC25C is predominantly expressed in the G_2 phase. CDC25B is over-

expressed in more than 70% of gastric cancers regardless of histological type and is closely correlated with tumour invasion and nodal metastasis (Kudo *et al.*, 1997). On the other hand, only 2% of gastric adenomas overexpress CDC25B. However, no gene amplification of *CDC25B* has been found in any gastric cancer. In 38% of gastric cancers, CDC25A is overexpressed but CDC25C is at very low or undetectable levels. Thus, the overexpression of CDC25B in tumour cells may stimulate progression of gastric cancer.

With regard to negative-cell cycle regulators, the p53-inducible CDK inhibitor p21 is associated with the senescence of non-neoplastic gastric epithelial cells (Harper *et al.*, 1993). In neoplastic lesions, the expression of p21 is seen in 78% of gastric adenomas and 76% of gastric adenocarcinomas regardless of *p53* gene mutation, suggesting that a *p53*-independent pathway is substantially involved in the induction of *p21* in gastric tumours (Yasui *et al.*, 1996a). In fact, the growth inhibition of transforming growth factor (TGF)-β or retinoic acid is associated with *p53*-independent induction of *p21* in a gastric cancer cell line (Akagi *et al.*, 1996). Moreover, the strong expression of p21 in cancer cells is frequently observed in advanced cancers and nodal metastasis, whereas there is no inverse correlation between p21 expression and proliferative activity measured by Ki-67. These findings indicate overall that the proliferative activity of gastric cancer cells is not solely dependent on control of the cell cycle by p21. In addition, mutation of the *p21* gene is exceptional in gastric cancer (Akama *et al.*, 1996a) and a codon 31 polymorphism does not affect the expression levels of p21 (Akama *et al.*, 1996b).

p27, a member of the cip/kip family of CDK inhibitors, binds to a wide variety of cyclin/CDK complexes and inhibits kinase activity. We have found that growth suppression of interferon-β is associated with the induction of p27 in a gastric cancer cell, TMK-1 (Kuniyasu *et al.*, 1997a). More importantly, reduction in p27 expression is frequently seen in advanced gastric cancers, whereas p27 is well preserved in 90% of gastric adenomas and 85% of early cancers (Yasui *et al.*, 1997). Gastric adenomas with reduction or loss of p27 are capable of developing into malignancies. Reduced expression of p27 significantly correlates with depth of tumour invasion and nodal metastasis. Moreover, metastatic tumour cells in lymph nodes express p27 at lower levels than do cells in primary tumours, suggesting that tumour cells with reduction or loss of p27 may selectively metastasize to lymph nodes or distant organs (Yasui *et al.*, 1999a). The expression of p27 in gastric cancer is inversely correlated with the expression of cyclin E (Igaki *et al.*, 1995). Loss of p27 function and gain of cyclin E evidently stimulate progression and metastasis of gastric carcinomas. Reduction in p27 expression occurs at post-translational levels, resulting from ubiquitin-mediated proteosomal degradation rather than genetic abnormalities (Yasui *et al.*, 1999a).

Deletion or mutations of the *p16* gene are uncommon in primary gastric carcinomas (Igaki *et al.*, 1995; Lee *et al.*, 1997; Gunther *et al.*, 1998), but homozygous deletion of this gene has been found in two of eight gastric cancer cell lines and lack of p16 protein expression in five of eight gastric cancer cell lines (Akama *et al.*, 1996b). Another mechanism of *p16* gene silencing is hypermethylation of the 5′CpG island (Merlo *et al.*, 1995). Reduced expression of p16 protein, probably by gene methylation, is found in about 20% of primary gastric cancers regardless of their histological type (Yasui *et al.*, 1996b). In particular, loss of p16 protein is often seen in advanced cancers with nodal metastasis. Loss of p16 and p27 proteins may be associated with the progression of gastric carcinoma. Chen *et al.* (1997b) reported that aberrant RNA transcripts of the *p16* gene is noted in 30–45% of primary gastric cancers.

Iida *et al.* (2000) reported that the *p14* (*ARF*) gene is more frequently inactivated by LOH or DNA methylation in diffuse-type gastric cancer than in those of the intestinal type, suggesting that alterations of *p14* (*ARF*) may be involved in diffuse-type gastric carcinogenesis.

Major alterations in the *Rb* gene are also infrequent in primary gastric cancers (Constancia *et al.*, 1994). All primary tumours and all gastric cancer cell lines express pRb (Akama *et al.*, 1996b).

An important downstream target of cyclin/CDKs at the G1/S transition is a family of E2F transcription factors. Gene amplification of *E2F-1* is seen in 4% of gastric cancers and 25% of colorectal cancers. Overexpression of E2F is found in 40% of primary gastric carcinomas (Suzuki *et al.*, 1999). Moreover, E2F and cyclin E tend to be co-expressed in gastric cancer. In contrast, 70% of gastric cancers exhibit lower levels of E2F-3 expression than corresponding non-neoplastic mucosas. These results suggest that gene amplification and anomalous expression of the *E2F* gene may permit the development of gastric cancer.

Cell-adhesion molecules and metastasis-related genes

Cell-adhesion molecules may also work as tumour suppressors. Mutations in the *E-cadherin* gene have been reported to occur preferentially in 50% of diffuse gastric carcinomas (Becker *et al.*, 1994). *E-cadherin* gene mutation is found in the diffuse component of mixed gastric carcinomas composed of both intestinal and diffuse types (Machado *et al.*, 1999). The results of Handschuh *et al.* (1999) indicate that *E-cadherin* mutations affecting exons 8 or 9 induce the scattered morphology, decrease cellular adhesion and increase cellular motility of diffuse gastric cancers. The mutations are even detected in intramucosal carcinoma (Muta *et al.*, 1996). *E-cadherin* germline mutations in familial gastric cancer have been reported since 1998, but their frequency is extremely rare (Guilford *et al.*, 1998; Iida *et al.*, 1999; Keller *et al.*, 1999; Yoon *et al.*, 1999). Kawanishi *et al.* (1995) found that a diffuse gastric carcinoma cell line, HSC-39, contained a mutation of the *β-catenin* gene. Moreover, Caca *et al.* (1999) reported that *β-* and *γ-catenin* mutations but not E-cadherin inactivation brought about constitutive Tcf transcriptional activity in gastric and pancreatic cancer cells. In addition to genetic alterations in *E-cadherin* and *β-catenin*, crosstalk between β-catenin and receptor tyrosine kinases including c-*met*, epidermal growth factor (EGF) receptor and c-*erb*B2 takes place in gastric cancer cells *in vitro* and *in vivo*, leading to diffuse spreading or scattering of gastric cancer cells (Ochiai *et al.*, 1994; Shibata *et al.*, 1996). These results indicate that genetic and epigenetic alterations in E-cadherin and catenins are involved in the development and progression of diffuse and scirrhous-type gastric cancers.

The *CD44* gene contains at least 20 exons, 12 of which can be alternatively spliced to make up a wide variety of molecular variants (Cooper *et al.*, 1992; Matsumura & Tarin, 1992). We have found that expression of abnormal CD44 transcripts, including exon 11, is frequently associated with primary gastric carcinomas and metastatic tumours (Yokozaki *et al.*, 1994). Moreover, the pattern of abnormal CD44 transcripts in the tumours differs between intestinal and diffuse gastric cancers. More importantly, all gastric cancer tissues and gastric cancer cell lines show overexpression of abnormal CD44 transcripts containing the intron 9 sequence (Higashikawa *et al.*, 1996), suggesting that the abnormal CD44 transcript containing the intron 9 sequence is presumably an effective biomarker for early detection of gastric cancers. Sixty per cent of gastric intestinal metaplasias express CD44 variants containing an intron 9 sequence; normal gastric mucosa does not express these variants (Yoshida *et al.*, 1995).

Osteopontin (OPN), also termed Eta-1 (early T-lymphocyte activation-1), which is a reported protein ligand of CD44, is overexpressed in 73% of gastric carcinomas (Weber *et al.*, 1996). The co-expression of OPN and CD44v9 in tumour cells correlates with the degree of invasion of lymphatic vessels or distant lymph node metastasis in diffuse gastric cancer (Ue *et al.*, 1998). In particular, clustering of the tumour cells in lymphatic vessels shows strong co-expression of OPN and Cd44v9. Therefore, mutual interactions between OPN and CD44v9 on the tumour cells may be used by CD44-bearing diffuse gastric carcinomas to promote lymphogenous metastasis.

A candidate suppressor gene related to metastasis, *nm*23, encodes nucleoside diphosphate kinase which may activate c-*myc* transcription factor. Although LOH of the *nm*23 gene in gastric cancer is rare, the reduced expression of *nm*23, presumably as a result of epigenetic mechanisms, is frequently associated with metastasis of gastric

cancer (Nakayama *et al.*, 1993). In addition to *nm*23, galectin-3 (known as lactoside-binding lactin L-31), which belongs to a family of galactoside-binding proteins, is frequently overexpressed in primary tumours and liver metastases of gastric cancer of the intestinal type (Lotan *et al.*, 1994). This higher expression of galectin-3 in gastric cancers and metastases implicates this lectin in the metastatic phenotype.

Amplification of c-*met* or K-*sam* in gastric cancer evidently contributes to progression and peritoneal invasion of diffuse gastric carcinoma. In addition, peritoneal dissemination requires LOH of 7q. Our study on deletion mapping of 7q has already demonstrated that LOH at the D7S95 locus is frequently associated with peritoneal dissemination (Kuniyasu *et al.*, 1994a). The D7S95 locus may contain a candidate suppressor gene for the progression and metastasis of gastric cancer.

Genetic instability

Two types of genetic instability involved are microsatellite instability (MSI) and chromosomal instability. MSI is caused by altered DNA mismatch repair. MSI has been found in 15–39% of sporadic gastric carcinomas worldwide (Semba *et al.*, 1996; Yokozaki *et al.*, 1999b). Gastric carcinomas with a high frequency of MSI (MSI-H) can be divided into two subtypes, intestinal and diffuse carcinomas, each of which has specific clinicopathological characteristics. Intestinal-type gastric cancers with MSI-H are often seen in patients over 73 years of age and often occur in the *antrum pylori*. They are frequently associated with abundant lymphoid infiltration, a putative favourable prognosis, and multiple tumours (Wu *et al.*, 1998; Leung *et al.*, 1999). Hypermethylation of the *hMLH1* gene promoter occurs in over 70% of cases with this type of gastric cancer and is often associated with down-regulation or loss of *hMLH1* (Fleisher *et al.*, 1999; Leung *et al.*, 1999). This evidence indicates that MSI-H in intestinal-type gastric cancer is mostly due to epigenetic inactivation of the *hMLH1* gene.

On the other hand, diffuse-type gastric cancers with MSI-H occur mostly in patients under 35 years of age, and are often accompanied by scirrhous-type carcinoma with diffusely productive fibrosis (Semba *et al.*, 1998). However, diffuse-type gastric cancers harbour no germline mutation of *hMLH*1 and *hMSH2* and no alteration at *BAT-RII*. This type of gastric cancer is frequently associated with LOH on chromosome 17q21, including the *BRCA1* gene. However, we have found no mutation of the *BRCA1* gene. This raises two possibilities: (1) chromosome 17q12–21, including the *BRCA1* locus, may contain a candidate tumour suppressor gene; (2) allelic loss of the *BRCA1* gene may be linked to frequent genetic instability in young patients with gastric cancer.

Microsatellite instability at the locus D1S191 (chromosome 1q) is found in 46% of intestinal gastric cancers but not in diffuse-type gastric carcinomas. Microsatellite alteration at the same locus is also seen in 26% of incomplete-type intestinal metaplasias adjacent to primary gastric cancers. Moreover, an identical pattern of microsatellite alteration at the locus D1S191 is detected in both intestinal-type adenocarcinoma and the adjacent intestinal metaplasia, suggesting the sequential development of intestinal adenocarcinoma from incomplete intestinal metaplasia (Hamamoto *et al.*, 1997). The results described above indicate that MSI at the D1S191 locus is one of the early events in the multistep process of stomach carcinogenesis.

Chromosomal instability leading to DNA aneuploidy is also an underlying factor in stomach carcinogenesis. Telomere length is necessary for maintaining chromosomal stability. Recent evidence indicates that in the absence of telomerase, telomere shortening can produce telomere dysfunction that causes both DNA breaks and chromosome gain or loss (Chin *et al.*, 1999; Hackett *et al.*, 2001). Therefore, telomere dysfunction may initiate chromosomal instability in tumorigenesis. Conversely, telomerase can inhibit chromosomal instability (Hackett *et al.*, 2001). Most intestinal carcinomas have remarkably shortened telomere length, associated with high levels of telomerase activity and significant expression of human telomerase reverse transcriptase (hTERT) (Tahara *et al.*, 1995; Yasui *et al.*, 1998). More importantly, over 50% of intestinal metaplasias, as well as adenomas, express low levels of telo-

merase activity equivalent to about one-tenth of the activity in gastric carcinomas (Yasui *et al.*, 1999b).

Immunohistochemistry shows that the hTERT protein is strongly expressed in the nuclei of the tumour cells of all carcinomas but weakly expressed in the nuclei of epithelial cells of intestinal metaplasia and gastric adenoma and in normal fundic mucosa (Yasui *et al.*, 1998). Thus, hTERT-positive epithelial cells in the above precancerous lesions and normal gastric mucosa may be viewed as epithelial 'stem cells'. Moreover, the prevalence of *H. pylori* infection in gastric mucosa correlates well with the grade of intestinal metaplasia and the levels of hTERT and of telomerase activity; the latter is frequently associated with hyperplasia of hTERT-positive epithelial cells (Kuniyasu *et al.*, 1997b; Yasui *et al.*, 1999b). These observations indicate that *H. pylori* infection may be a strong trigger for hyperplasia of hTERT-positive cells in intestinal metaplasia, followed by increased telomerase activity and telomere reduction. Hyperplasia of hTERT-positive cells caused by *H. pylori* may induce 'chronic mitogenesis' which can facilitate increased mutagenesis. In fact, DNA hypermethylation at the D17S5 locus, *pS2* loss, abnormal CD44 transcripts, CA repeat instability at the D1S19 locus, and *APC* and *p53* mutations, all of which are commonly seen in intestinal gastric cancer, occur in over 30% of incomplete intestinal metaplasias (Tahara, 1998).

These data all indicate that telomere reduction and hTERT overexpression due to stem-cell hyperplasia are very early events in the multistep development of intestinal-type gastric cancer, followed by the above-mentioned epigenetic and genetic alterations. The frequent development of intestinal-type gastric cancer in elderly patients with *H. pylori* infection suggests that this type of gastric cancer is a disease of a 'chronically afflicted genome' rather than a genetic disease.

Telomerase-negative gastric carcinomas are only of the diffuse type, not the intestinal type, although their incidence is 13–15% (Tahara *et al.*, 1995). Diffuse-type gastric cancers occasionally harbour extremely long telomere length and have genetic alterations that are different from those of carcinomas of the intestinal type. Hence, a telomerase-independent or alternative mechanism may be involved in neoplastic transformation and immortalization of the cells of some diffuse-type gastric cancers.

Mutations in the *p53* gene are also implicated in chromosomal instability. Recently Kaplan and coworkers found that mutation in *APC* may be responsible for chromosomal instability in colon cancer (Kaplan *et al.*, 2001). APC protein directly binds to a kinetochore protein and is an avid in-vitro substrate of the mitotic check-point protein Bub1 (Pellman, 2001). It remains to examine whether gastric cancer cells carrying a truncated *APC* gene are defective in chromosome segregation. Mutations of the *hBub1* gene have been reported in colon cancers (Cahill *et al.*, 1998). However, there is no mutation in the *hBub1* gene in gastric carcinomas (Shigeishi *et al.*, 2001).

Growth factors and cytokines

Gastric cancer cells express a broad spectrum of growth factors, cytokines or both, including TGF-α, TGF-β1, EGF, amphiregulin (AR), cripto, heparin binding (HB)-EGF, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) II, basic fibroblast growth factor (bFGF), interleukin (IL)-1α, IL-6, IL-8 and OPN (Tahara, 1993; Tahara *et al.*, 1994, 1996b; Tahara, 1997). These growth factors and cytokines function as autocrine, paracrine and juxtacrine modulators of the growth of cancer cells, and they organize the complex interaction between cancer cells and stromal cells which plays a key role in morphogenesis, invasion, neovascularization and metastasis. Interestingly, the expression of these growth factors, cytokines or both by cancer cells differs in the two histological types of gastric carcinoma. The EGF family, including EGF, TGF-α and cripto, is commonly overexpressed in intestinal-type gastric carcinoma, whereas TGF-β, IGF-II and bFGF are predominantly overexpressed in the diffuse type (Tahara *et al.*, 1999). Co-expression of EGF/TGF-α, EGF receptor and cripto correlates well with the biological malignancy of gastric cancer, because these factors induce metalloproteinases (Yasui *et al.*, 1988; Yoshida *et al.*, 1990; Kuniyasu

et al., 1994b). Overexpression of cripto is frequently associated with intestinal metaplasia and gastric adenoma (Kuniyasu *et al.*, 1991).

AR, a member of the EGF family which is overexpressed in more than 60% of gastric carcinomas regardless of histological type (Kitadai *et al.*, 1993a), works as an autocrine growth factor and induces the expression of AR itself, TGF-α and EGF receptors by gastric cancer cells (Akagi *et al.*, 1995). Overexpression of the EGF family in gastric cancer usually does not accompany gene amplifications. The relative expression levels of positive transcription factor, Sp-1, and negative transcription factor, GC factor, may regulate gene expression of these growth factors and receptors (Kitadai *et al.*, 1993b).

IL-1α is a cytokine mainly produced by activated macrophages and mediates many of the local and systemic responses to infection and inflammation (Dinarello, 1992). It is also produced by gastric cancer cells. We have found that IL-1α evidently acts as an autocrine growth factor for gastric carcinoma cells and plays a pivotal role as a trigger for induction of EGF and EGF receptor expression (Ito *et al.*, 1993). The expression of IL-1α by tumour cells is induced by either IL-1α, EGF or TGF-α, while IL-1α up-regulates the expression of TGF-α and EGF receptor by tumour cells themselves, indicating that an intimate interplay between IL-1α and the EGF/receptor system stimulates the growth of gastric cancer.

In addition to IL-1α, IL-6 is also an autocrine growth stimulator for gastric cancer cells. The expression of IL-1α by tumour cells is induced by IL-6, while IL-1α increases the expression of IL-6 by tumour cells themselves (Ito *et al.*, 1997).

Stromal cells, especially fibroblasts stimulated by growth factors or cytokines such as IL-α, TGF-α and TGF-β, secrete HGF/SF (hepatocyte growth factor/scatter factor), which can function in a paracrine manner as a morphogen or motogen of tumour cells. For example, in the case of a clone maintaining expression of cell-adhesion molecules, HGF/SF promotes tubular formation of tumour cells, resulting in intestinal-type gastric cancer. Conversely, in the case of a clone with reduced expression of cell-adhesion molecules, HGF/SF can act as a motogen and induce scattering of tumour cells, resulting in diffuse gastric cancer (Tahara, 1993; Yokozaki *et al.*, 1997). Our recent findings suggest that interaction between c-*met* overexpressed in tumour cells and HGF/SF from stromal cells is related to the morphogenesis and progression of gastric cancer *in vivo*.

The negative growth factor TGF-β1 is commonly overexpressed in gastric carcinoma, particularly in diffuse-type carcinoma with diffusely productive fibrosis (Yoshida *et al.*, 1989). However, most human gastric cancer cells have escaped from TGF-β-induced growth inhibition at the receptor or post-receptor levels. TGF-β inhibited the growth of only one (TMK-1) of seven gastric carcinoma cell lines; this inhibition is associated with p53-independent induction of p21 which induces suppression of cyclin-dependent kinase activity, reduced phosphorylation of Rb and a decrease in cyclin A (Ito *et al.*, 1992a; Akagi *et al.*, 1996). Various mutations in the *TGF-β* receptor type II (RII) gene have been reported in gastric cancer. One type of mutation in the *TGF-βRII* gene is mutation in the polyA tract (i.e. deletion or insertion of 1–2 bases) that frequently occurs in the hereditary non-polyposis colon cancer syndrome (Markowitz *et al.*, 1995) and in gastric carcinoma with MSI-H (Yokozaki *et al.*, 1999b). Another type of mutation in the *TGF-βRII* gene involves abnormal amplification and truncation of the gene (Yang *et al.*, 1999). However, we have not seen genetic alterations of the *TGF-βRII* gene in any gastric carcinoma cell lines. Moreover, results of a study on expression of TGF-βRI in TGF-β-resistant gastric cancer cell lines that contain no discernible alteration in the *TGF-βRII* gene suggest that hypermethylation of a CpG island in the 5′ region of the *TGF-βRI* gene is involved in another potentially important mechanism of escape from negative growth control by TGF-β (Kim *et al.*, 1999). We have already found that most gastric carcinomas show reduced levels of TGF-βRI and that this correlates well with the depth of tumour invasion (Ito *et al.*, 1992b).

A large number of angiogenic factors have been identified in human malignancy. Among them are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and IL-8, which

are derived from tumour cells and participate mainly in neovascularization within gastric carcinoma tissues. We have shown that all eight gastric cancer cell lines secrete VEGF into conditioned media (Yamamoto *et al.*, 1998). EGF or IL-1α up-regulates VEGF expression by tumour cells, whereas interferon-γ down-regulates it. Moreover, VEGF promotes angiogenesis and the progression of gastric carcinomas, especially carcinomas of the intestinal type (Takahashi *et al.*, 1996). On the other hand, bFGF produced by tumour cells is frequently associated with angiogenesis and extensive fibrosis in diffuse gastric carcinomas, particularly those of the scirrhous type (Tanimoto *et al.*, 1991).

IL-8, a member of the CXC chemokine family, induces haptotactic migration and proliferation of melanoma cells and angiogenesis. More importantly, gastric carcinoma cell lines express mRNA and protein for IL-8 and IL-8 receptors (IL-8RA and IL-8RB) (Kitadai *et al.*, 1998, 2000). More than 80% of gastric carcinomas co-express IL-8 and IL-8 receptors; this co-expression correlates directly with tumour vascularity and disease progression. IL-8 enhances the expression of EGF receptor, type IV collagenase (metalloproteinase (MMP)-9), VEGF and IL-8 mRNA itself by gastric cancer cells, whereas IL-8 decreases expression of E-cadherin mRNA. In addition, IL-8 also increases MMP-9 activity and the ability of gastric cancer cells to invade through Matrigel. Altogether, IL-8 may play an important role in the growth and progression of gastric carcinoma by autocrine and paracrine mechanisms.

Factors associated with increased incidence of gastric cancer

Three major factors, including environmental factors, host factors and genetic factors, cooperatively affect the genesis of gastric cancer (Table 2). Of these, environmental factors are the most important, as diet and cigarette smoking are primary offenders; in particular, the presence of carcinogens such as *N*-nitroso compounds and benzo[*a*]pyrene is directly linked to carcinogenesis. As already described, the mutation spectrum of the *p53* gene differs between intestinal-type and diffuse-type gastric cancers, suggesting that different carcinogens may be implicated in the two types of gastric carcinogenesis (Yokozaki *et al.*, 1997). Palli *et al.* (2001) found that the risk of MSI-H gastric cancer was positively associated with high consumption of red meat and meat sauce, and negatively associated with consumption of white meat.

Table 2. Factors associated with increased incidence of gastric carcinoma

Environmental factors	Diet (nitrites derived from nitrates, smoked and salted food, pickled vegetables, lack of fresh fruit and vegetables) Cigarette smoking
Host factors	*H. pylori* infection (chronic gastric and intestinal metaplasia) Partial gastrectomy Barrett esophagus
Genetic factors	Hereditary diffuse gastric cancer Hereditary non-polyposis colon cancer DNA polymorphism Genetic instability

With regard to host factors, meta-analysis of the relationship between *H. pylori* infection and gastric cancer has indicated that *H. pylori* infection is associated with a twofold increased risk of gastric cancer (Huang *et al.*, 1998; Eslick *et al.*, 1999). Younger *H. pylori*-infected patients have a higher relative risk for gastric cancer than older patients. *H. pylori* infection is equally associated with intestinal-type and diffuse-type gastric cancers (Huang *et al.*, 1998). In fact, the findings in a Mongolian gerbil model of stomach carcinogenesis indicate that *H. pylori* infection promotes stomach carcinogenesis induced by chemical carcinogens, and that histological types of gastric carcinoma may depend on the concentration of chemical carcinogens rather than on *H. pylori* infection (Shimizu *et al.*, 1999). Eradication of the bacteria evidently decreases the incidence of gastric carcinomas in the Mongolian gerbil model (Shimizu *et al.*, 2000).

H. pylori infection produces reactive oxygen and nitrogen species that cause DNA damage, followed by chronic gastric and intestinal meta-

plasia (Correa *et al.*, 1997). Goto *et al.* (1999) reported that the expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in the gastric mucosa was significantly high in *H. pylori*-infected patients who developed gastric cancer at least 2 years after the initial biopsies. These findings suggest that high production of iNOS and nitrotyrosine in the gastric mucosa by *H. pylori* may contribute to gastric carcinogenesis.

Cyclooxygenase-2 (Cox-2) expression is also induced by *H. pylori* infection (Sung *et al.*, 2000). Successful eradication of *H. pylori* leads to down-regulation of Cox-2 in the epithelial and stromal cells. High expression of Cox-2 mRNA, protein and enzymatic activity is detected preferentially in the tumour cells of intestinal-type gastric cancer (Saukkonen *et al.*, 2001). Loss of Cox-2 promoter methylation may enhance Cox-2 expression and promote gastric carcinogenesis associated with *H. pylori* infection (Akhtar *et al.*, 2001).

It should not be forgotten that in Japan the annual incidence of gastric cancer is about 100 000, accounting for 0.16% of 60 million individuals with *H. pylori* infection. Moreover, analysis of chromosomal aberrations in gastric cancer shows that they do not differ between *H. pylori*-related and non-related gastric cancers (van Grieken *et al.*, 2000). Genetic factors play a critical role in susceptibility to stomach carcinogenesis (Table 3).

Prinz *et al.* (2001) reported that cagA+/vacAs1+ strains of *H. pylori* that are blood-group antigen-binding adhesion (BabA2)-positive are associated with activity or chronicity of gastritis. Adherence of *H. pylori* via BabA2 may play a key role for efficient delivery of VacA and CagA.

In addition to *H. pylori* strains, DNA polymorphism including HLA, MUC1 (Carvalho *et al.*, 1997), T-cell helper 1 and IL-1β has been reported to be associated with an increased risk of both atrophic gastritis induced by *H. pylori* and gastric cancer (El-Omar *et al.*, 2000). More excitingly, Magnusson *et al.* (2001) found that distinct HLA class II DQ and DR alleles are associated with the development of gastric cancer and infection with *H. pylori*. The DQA1*0102 is associated with protection from *H. pylori* infection, whereas the DRB*1601 is associated with cancer development, particularly *H. pylori*-negative diffuse gastric cancer. These host genetic factors may determine why some individuals infected with *H. pylori* develop gastric cancer while others do not. However, these studies need confirmation by a large number of prospective investigations in each of the countries concerned.

Table 3. Susceptibility to disease caused by *H. pylori*

Strain of *H. pylori*	CagA+/VacAs1+strains that are BabA2-positive (Printz *et al.*, 2001)
Genetic factors	*HLA* polymorphism: *HLA*-DQA1 genetic typing *MUC1* polymorphism T-cell helper 1 phenotype IL-1β polymorphism *HLA* DR and DQ alleles: DQA1*0102 is associated with protection from infection by *H. pylori*, whereas DRB*1601 is associated with cancer development, particularly *H. pylori*-negative diffuse type (Magnusson *et al.*, 2001).

Conclusion

Overall, the observations on the molecular events of gastric cancer may provide supporting evidence for our working hypothesis that there are two distinct major genetic pathways for stomach carcinogenesis (Figure 1). Genetic and epigenetic alterations found in two types of gastric cancer are summarized in Table 1. Among them, genetic instability including MIS and telomere reduction and immortality (activation of telomerase and expression of hTERT) are implicated in an initial step of stomach carcinogenesis. In the multistep process of intestinal-type gastric carcinogenesis, infection with *H. pylori* may be a strong trigger for hyperplasia of hTERT-positive stem cells in intestinal metaplasia. Genetic instability and hyperplasia of hTERT-positive stem cells may precede replication error at the D1S191 locus, DNA hypermethylation at the D17S5 locus, *pS2* loss, *RARβ* loss, CD44 abnormal transcripts and *p53* mutation, all of which accumulate in at least 30% of incomplete intestinal metaplasias. All of these epigenetic and genetic alterations are

common events in intestinal-type gastric cancer. Incomplete intestinal metaplasia that contains an accumulation of the above multiple molecular events – that is, 'metaplastic dysplasia' – may be viewed as a bud of intestinal-type gastric cancer at genetic and epigenetic levels. An adenoma→carcinoma sequence is found in about 20% of gastric adenomas with *APC* mutations. In addition to these events, *p53* mutation and LOH, reduced p27 expression, cyclin E expression and presence of c-*met* 6.0-kb transcripts allow malignant transformation from the precancerous lesions to intestinal-type gastric cancer. *DCC* loss, *APC* mutations, 1q LOH, *p27* loss, reduced TGF-βRI expression, reduced nm23 expression and c-*erb*B gene amplification are implicated in the progression and metastasis of intestinal-type gastric cancer. Another pathway for carcinogenesis of intestinal-type gastric cancer involves LOH and abnormal expression of the *p73* gene that may be responsible for the development of foveolar-type gastric cancers with pS2 expression.

On the other hand, LOH at chromosome 17p, mutation or LOH of *p53* and mutation or loss of *E-cadherin* are preferentially involved in the development of diffuse gastric cancers. In addition to these changes, gene amplification of K-*sam* and c-*met* and *p27* loss as well as reduced nm23 obviously confer progression, metastasis and diffusely productive fibrosis.

Mixed gastric carcinomas composed of intestinal and diffuse components exhibit some but not all of the molecular events described for each of the two types of gastric cancer.

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